ICOZ Recid PCT/PTO 1 0 DEC 2001 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE FORM PTO-1390 (Modified) 1908-006-27 TRANSMITTAL LETTER TO THE UNITED STATES U.S APPLICATION NO. (IF KNOWN, SEE 37 CFR 10/009344DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371 INTERNATIONAL FILING DATE PRIORITY DATE CLAIMED INTERNATIONAL APPLICATION NO 9 JUNE 1999 9 JUNE 2000 PCT/US00/15820 TITLE OF INVENTION CHROMATOGRAPHIC DETERMINATION OF P-GLYCOPROTEIN REACTIVE LIGANDS APPLICANT(S) FOR DO/EO/US WAINER, Irving and ZHANG, Yanxiao Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information This is a FIRST submission of items concerning a filing under 35 U S.C. 371. This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 2. This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), 3.  $\boxtimes$ (6), (9) and (24) indicated below. The US has been elected by the expiration of 19 months from the priority date (Article 31).  $\boxtimes$ 4. A copy of the International Application as filed (35 U.S.C. 371 (c) (2)) 5.  $\boxtimes$ is attached hereto (required only if not communicated by the International Bureau). a. 🗆 has been communicated by the International Bureau. b. 🗆 is not required, as the application was filed in the United States Receiving Office (RO/US). c. 🖾 An English language translation of the International Application as filed (35 U.S.C 371(c)(2)). a. 🗆 is attached hereto. has been previously submitted under 35 U.S.C. 154(d)(4). b. 🗆 Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)) are attached hereto (required only if not communicated by the International Bureau). a. 🗆 have been communicated by the International Bureau. b. 🗆 have not been made; however, the time limit for making such amendments has NOT expired. c. 🗆 have not been made and will not be made. d 🔯 An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).  $\Box$ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). 9. An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)). 10. A copy of the International Preliminary Examination Report (PCT/IPEA/409). X 11. A copy of the International Search Report (PCT/ISA/210).  $\bowtie$ 12. Items 13 to 20 below concern document(s) or information included: An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 13. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 14. A FIRST preliminary amendment. 15. A SECOND or SUBSEQUENT preliminary amendment. 16. 

- 17. A substitute specification.
- 18. 

  A change of power of attorney and/or address letter.
- 19. A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S C. 1.821 1.825.
- 20. 

  A second copy of the published international application under 35 U.S.C. 154(d)(4)
- 21. 

  A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
- 23. Other items or information:

Copy of the published International Application (WO 00/75179)
White Advance Serial Number Postcard

JC07,Rec'd.PCT/PTO, ,1-0, DEC 2001 U.S. APPLICATION NO INTERNATIONAL APPLICATION NO PCT/US00/15820 1908-006-27 The following fees are submitted:. CALCULATIONS PTO USE ONLY 24. BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ...... \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . . . . . . . \$740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).... \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT = \$710.00 Surcharge of \$130.00 for furnishing the oath or declaration later than \$130.00 months from the earliest claimed priority date (37 CFR 1.492 (e)). RATE NUMBER EXTRA NUMBER FILED **CLAIMS** 0 х \$18.00 \$0.00 Total claims 10 -20 =\$0.00 0 \$84 00 Independent claims - 3 = \$0.00 Multiple Dependent Claims (check if applicable) TOTAL OF ABOVE CALCULATIONS \$840.00 Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are \$420.00 reduced by 1/2. \$420.00 SUBTOTAL Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)). □ 30 □ 20 \$0.00 TOTAL NATIONAL FEE \$420.00 Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). \$0.00 \$420.00 TOTAL FEES ENCLOSED Amount to be: refunded charged \$ \$420.00 to cover the above fees is enclosed.  $\boxtimes$ A check in the amount of to cover the above fees. 50-1442 in the amount of Please charge my Deposit Account No. b. A duplicate copy of this sheet is enclosed. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment  $\boxtimes$ c. A duplicate copy of this sheet is enclosed. to Deposit Account No. 50-1442 Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card d. information should not be included on this form. Provide credit card information and authorization on PTO-2038. NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status. SEND ALL CORRESPONDENCE TO: Supervisor, Patent Prosecution Services PIPER MARBURY RUDNICK & WOLFE, LLP KELBER, Steven B. 1200 Nineteenth Street, NW **NAME** Washington, DC 20036-2412 30.073 REGISTRATION NUMBER 10 DECEMBER 2001 (Monday) DATE

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CHROMATOGRAPHIC DETERMINATION OF P-GLYCOPROTEIN REACTIVE LIGANDS

# Field of the Invention:

This invention relates to immobilization of transporters on a support in a liquid chromatographic system.

# **Background of the Invention:**

The combinatorial synthesis of chemical libraries has created an enormous pool of possible new drug candidates. Indeed, synthetic capabilities have outstripped the ability to determine corresponding biological activity. An initial step in the resolution of this problem has been the development of microliter plates which contain immobilized receptors/ antibodies. The use of these plates can rapidly reduce the number of possible candidates in a combinatorial pool from thousands to hundreds. However, assignment of relative activity within the reduced pool of compounds remains a slow and repetitive process.

The relationship between basic pharmacological processes and liquid chromatographic (LC) studies have been emphasized by the inclusion of biomolecules as active components of chromatographic systems. A wide variety of immobilized biopolymer-based LC stationary phases (BP-SPs) have been developed using proteins, enzymes, cellulose and amylose, macrocyclic antibodies and liposomes. Indeed, it has been demonstrated that the chromatographic retention and selectivity of BP-SPs are related to the properties of the non-immobilized biopolymer. For example, retention of a compound on an SP column containing immobilized human serum albumin has been used to evaluate the binding properties of the compounds to proteins.

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P-glycoprotein (PGP) is a 170 to 180 kDA membrane transporter that acts as an ATP-driven drug efflux pump. The over-expression of PGP has been associated with multidrug resistance (MDR) in tumor cells and the MDR phenotype is a key factor in the failure of chemotherapeutic treatment of breast cancer. The MDR1 genes encode the PGP that is involved in the MDR phenotype. A breast tumor is two times more likely to express MDR1/PGP if it has been exposed to cytotoxic chemotherapy and almost three times more likely to be resistant to adriamycin *in vitro* if it expresses MDR1/PGP.

One approach to the development of therapeutic protocols to overcome MDR in breast cancer patients has concentrated on the inhibition of the PGP-mediated pump. For example, *in vitro* studies have demonstrated that the presence of verapamil in the incubation media increased the cytotoxicity of vinca alkaloids and anthracyline derivatives in MDR1/PGP tumor cell lines. However, a clinical trial combining verapamil and the vinca alkaloid VP16 and the anthracycline derivative adriamycin was not successful due to the cardiotoxicity of verapamil. Thus, the development of novel agents to reverse MDR1/PGP-mediated drug resistance remains a key objective in breast cancer research.

The functions of PGP have been studied using a variety of experimental formats, including detergent solutions, proteo-liposomes, membrane vesicles and native membranes. Only a few of these studies have concentrated on evaluation of ligand-binding parameters and the screening of pools of drug candidates for their PGP binding affinities presents a formidable task.

# **Summary of the Invention:**

The invention provides for liquid chromatographic stationary phases to which are immobilized transporters, especially P-glycoproteins. These stationary phases are useful for identifying compounds by a continuous liquid

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chromatographic process which provides for the characterization, isolation and/or identification of compounds that specifically bind to the immobilized transporter. Thereby, the invention provides continuous on-line processes for identifying and isolating agents that bind to transporters, especially p-glycoproteins.

Accordingly, the present invention provides substrate systems and methods for continuous on-line evaluation, identification and isolation of compounds which bind to PGP.

# **Description of the Invention:**

It is the purpose of this invention to provide means for immobilization of membrane transporters in order to study interactions of compounds therewith. The fundamental processes of drug action, absorption, distribution and receptor activation, are dynamic in nature and have much in common with the basic mechanisms involved in chromatographic distribution. Indeed, the same basic intermolecular interactions (hydrophobic, electrostatic and hydrogen bonding) determine the behavior of chemical compounds in both biological and chromatographic environments. These properties are clearly illustrated herein using a PGP-based stationary phase (SP) system.

Although receptors and transporters play an important role in drug activity and are key targets in combinatorial screens, they have not been included in LC systems.

The use of receptors on supports comprising the steps of:

- (a) immobilizing receptors on artificial membrane supports in a column,
- 25 (b) exposing the supports with the receptors to test agents at varying concentrations in a liquid chromatographic system,

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- (c) eluting the test agent from the column, and
- (d) evaluating the elution profile of the test materials from the column has previously been disclosed. Using this method, it was possible to evaluate the interaction of the test agent with the receptor. Following elution, it is possible to directly determine molecular structure by passing the elute through other testing devices such as a mass spectrometer.

The present invention is exemplified herein using a PGP-based LC stationary phase for the study of drug-PGP interactions. The method was assessed in studies of the binding affinity of vinblastine, verapamil and cyclosporin A on PGP. Based thereon, it is anticipated that the present invention should be suitable for study of other ligand interactions with PGP in a dynamic manner.

The PGP-based stationary phase can be prepared by embedding PGP in a phospholipid monolayer of an immobilized artificial membrane (AIM) HPLC stationary phase, creating the PGP-IAM. In a second approach, PGP can be reconstituted into the phospholipid layer of liposomes that are immobilized on Superdex 200 gel beads by using freeze-thawing methods (PGPLIP). The latter approach was originally developed for the immobilization of liposomes or liposomes containing human red cell glucose transporter in chromatographic stationary phase. (See Wallsten, et al., *Biochim. Biophys. Acta* 982 (1987) 47 or Yang, et al, *Anal. Biochem.* 218 (1994) 210.)

The PGP-IAM was used to determine the PGP binding affinities of the vinblastine, cyclosporin A and verapamil. The binding affinities were assessed using frontal chromatographic techniques. The rank order of the calculated K<sub>d</sub> values, i.e., highest affinity to lowest affinity, are consistent with previously reported values. The PGP-IAM was stable, reproducible and deemed a useful

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addition to the art relating to G-ligand interactions and provides means for the use of rapid on-line screening of new agents for the treatment of MDR1/PGP resistant tumors.

# **Brief Description of the Figures:**

Figure 1 shows elution profile of [<sup>3</sup>H]VB[1nm] in frontal chromatography based on the PGP IAM column (0.5 X 0.8 cm) in the absence and in the presence of doxorubicin (200 nm) (profile B) in the mobile phase. Tris -Hcl buffer [50 mm, pH 7.4], flow rate 0.4 ml/min.

Figure 2 is a frontal analysis of interaction of PGP with verapamil on an immobilized PGP-IAM column (0.5 X 0.8 cm). The elution profiles of 1.0 nM [ $^{3}$ H] verapamil in solution with 10, 40, 60, 200 and 400 $\mu$ M non-radioactive verapamil (from right to left). Running buffer is 50 mM Tris-HCl, pH 7.4 at a flow rate of 0.5 ml/min.

Figure 3 is a nonlinear regression analysis of verapamil interaction with PGP-IAM. The data is from frontal analysis measurements.

Figure 4 shows zonal affinity chromatographic profiles of 100  $\mu$ l of 23.5 nM [ $^{3}$ H] verapamil at a flow rate of 0.5 ml/min with 50 mM Tris-HCl, pH 7.4 buffer, (1) was from PGP-negative-IAM column, (2) was from IAM particles column, and (3) was from PGP-IAM column.

Figure 5 is a frontal affinity analysis of 1.0 nM [3H] cyclosporine A was in the sample alone, (B) 50 nM cold vinblastine was supplemented in the sample, (C) 3 mM ATP was in the sample and running buffer, (D) 100 nM cold vinblastine was added in the sample. The running buffer was 50 mM Tris-NCl, pH 7.4.

Figure 6 is a frontal affinity chromatographic analysis of 1 nM [<sup>3</sup>H] vinblastine with PGP-IAM on a column of 0.5 X 0.8 cm at a flow rate of 0.5

ml/min (A). It was obtained with 1.0 nM [<sup>3</sup>H] vinblastine only (B). 1.0 nM [<sup>3</sup>H] vinblastine supplemented with 3 mM ATP. The running buffer for both (A) and (B) was 50 mM Tris-IICl, pH 7.4 with 1.6% ethanol.

## **EXAMPLE 1**

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#### Materials and methods:

The following materials and methods were used in Example 1.

Preparation of immobilized P-glycoprotein based-HPLC stationary phases.

Immobilized P-glycoprotein (PGP) Artificial Membrane (IAM)

The cultured cells MDA436/LCC6MDR1 ( 20 x 106 cells) were harvested in 30 ml of PBS saline and homogenized for 20 sec with a Brinkmann Polytron homogenizer. The homogenates were centrifuged at 35,000 x g for 10 min and the pellets were suspended in 4 ml solubilization solution (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% CHAPS, 2 mM DTT, 5% glycerol) and stirred for 1 hr at 0° C.

Two hundred (200) mg of dried IAM particles was suspended in 4 ml receptor-detergent solution and stirred for 1 hour at 4 C. The mixture was dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA) for 72 hours at 4° C. The obtained PGP-IAM particles were washed with the buffer by centrifugation and packed in a glass column (id 0.5 cm).

# Reconstitution and immobilization of PGP on Superdex 200 gel beads

The cultured cells MDA436/LCC6MDR1 (20 x 10<sup>6</sup> cells) were harvested in 20 ml of PBS saline and homogenized for 20 sec with a Brinkmann Polytron homogenizer. The homogenates were centrifuged at 35,000 x g for 10 min and the supernatant was discarded. The pellets were suspended in 4 ml solubilization solution [50 mM Tris-HCl, pH 7.5 containing 1.4% octyl -D-glucopyranoside,

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20% glycerol, 1 mM Dithiothreitiol, 1 mM benzamidine and 0.4% phospholipid: *E.coli* bulk phospholipid: PC:PS:Cholesterol (60:17.5:10:12.5)] by stirring at 0° C for 40 minutes. Nonsoluble material was removed by centrifugation. The supernatant was applied on to a Sephadex G50 column which equilibrated with elution buffer (150 mM NaCl, 10 mM TrisHCl, 1 mM EDTA, 1 mM Benzamidine). The liposome fractions were collected and concentrated to 1 ml. The concentrated liposome solution was mixed with 50 mg dried Superdex 200 and kept in room temperature for 2 hours. The mixture of liposome and Superdex 200 was frozen at -75 C for 10 min and then thawed at 25 C for 10 min and the freeze-thaw cycle was repeated. The non-immobilized liposomes were removed by centrifugation and the resulting PGP-Superdex gel beads were packed in a LC column.

# Chromatographic analysis of binding affinity of 3H'VBL at PGP

The PGP-IAM column or PGP-Superdex 200 column was washed with buffer (150 mM NaCl, 10 mM Tris-HCl buffer, pH 7.4, 1 mM EDTA). The column was placed in a standard HPLC system. 10-40 ml of 3H VBL with different concentrations in buffer or 1 nM 3H-VBL plus 200 nM ADR in buffer were applied onto the columns at 0.4 ml/min. An on-line flow scintillation detector monitored the elution profile.

#### 20 Binchoninic Acid (BCA) Protein Assay

The PGP-IAM and PGP-Superdex 200 packing materials were collected and the supernatants were removed. The samples were diluted with 0.1 N of NAOH to 2 ml. A protein standard (0.2-25 g protein in 50 l) was prepared with Albumin standard (Pierce) and 20 ml of reagent A mixed with 0.4 ml of reagent B. The standards and samples (50 l each) were added to triplicate wells in a plate. 200 l of BCA reagents (A+B) (Pierce) was added in each well. The plate

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was incubated for 2h at room temperature and read in a spectrophotometer at 570 nm using the Softmax program for calculation of the protein amount.

#### Results:

The protein assay showed that for one milliliter of bed volume, about 170 mg proteins were immobilized on IAM column and about 10 mg proteins were immobilized on Superdex 200 column. The chromatographic results obtained with PGP-IAM column or PGP-Superdex 200 column indicated that the binding activity of PGP was retained after immobilization. For example, 3H-VBL was retarded on a PGP-IAM column (0.5 x 0.8 cm) and the retention volume was 13.3 ml at the concentration of 1 nM (profile A in Fig 6) at flow rate: 0.4 ml/min. When a displacer ligand, ADR (200 nM), was included in the mobile phase, the retention volume of 1 nM [3H]-VBL was decreased from 13.3 ml to 6.5 ml (profile B in Fig 1). This indicated that the retardation was partially due to the specific binding to saturable binding sites of PGP.

### Calculation of Kd value for VBL:

The retention volumes of 3H-VBL at the different concentration in frontal chromatography were used to calculate the Kd value. The obtained Kd value for 3H-VBL determined in this technique is  $19 \pm 20$  nM; that is consistent with the reported value,  $36 \pm 55$  nM. These results indicated that PGP-based chromatographic stationary phase can be used for the evaluation of PGP-substrate interactions.

### Calculation of the binding capacity of the PGP-SP:

Approximately 24 mg of protein was immobilized on 100 mg of IAM support (0.24 mg protein/mg IAM) and the support was packed into a 0.8 cm x 0.5 cm ID column {volume 0.2 ml}. When 1 nM VBL was pumped through the column, the retention volume was 13.3 ml indicating that the column

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retained 13.3 pmoles of the substrate before saturation or 0.133 pmoles VBL per mg PGP-SP. Since neither the PGP-SP nor the chromatographic conditions used in these experiments were optimized, the results indicate that a PGP-SP prepared and operated under optimum conditions will have sufficient capacity for screening of complex mixtures for PGP substrates.

# EXAMPLE 2:

# On-line screening of complex mixtures for PGP substrates

# Step 1: Initial Chromatographic Screen:

The Initial Chromatographic Screen has been described above.

# Step 2: Secondary Chromatographic Screen:

The primary fractions isolated in the Initial Chromatographic Screen and screened are concentrated under reduced pressure to a volume of approximately 0.5 ml and then introduced onto the PGP-SP LC/MS system described above showing the PGP-SP LC/MS system used as the Secondary Chromatographic Screen.

#### Chromatographic Step A:

The primary fraction is injected on the PGP-SP column and the switching valve is be set to direct the flow from the PGP-SP column to the storage container. This configuration of the system is maintained until the elution volume {or k'} of the least retained marker PGP-substrate {i.e., VBL, TAX, or ADR} has been reached. Since the previous studies have established the relationship between k' and Kd, this procedure directs all of the compounds in the primary fraction with a Kd less than that of the lowest marker PGP-substrate to the SC. These compounds will not be lost and will be available for subsequent analysis.

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#### Chromatographic Step B:

After the k' of the least retained marker PGP-substrate has been reached, the switching valve is rotated and the flow from the PGP-SP column is directed onto the C18 column for the analytical separation of the compounds eluted from the PGP-SP. Since the mobile phases employed on the PGP-SP primarily consists of aqueous buffers it is assumed that the compounds will not elute from the C18 under these conditions and will concentrate at the top of the analytical column. Once the compounds have been washed off the PGP-SP {the initial cutoff is set at low-column volumes after the k' of the highest retained marker PGP-substrate}, the switching valve is rotated to its initial position allowing for a recharging of the PGP-SP) and a second HPLC pump is automatically brought on-line to the C18 column and a gradient elution program begun.

# Chromatographic Step C:

The gradient elution program elutes the compounds from the C18 column and into the pre-MSD splitting valve. A portion of the compounds eluted from the C18 column is directed through the splitter to collection tubes placed in an automatic fraction collector and stored for use. The remaining portion of the compound is directed to the HP 1100 MSD and the corresponding mass spectrum obtained. The automatic fraction collector is controlled by a signal from the MSD. When the slope of the total ion current detected by the MSD changes in a positive direction, the automatic fraction collector moves from waste to the next available collection tube and when the ion current returns to a preset level, the automatic fraction collector returns to waste.

#### Step 3: Additional Chromatographic Screens

Once the fractions collected from the Secondary Chromatographic Screen have been assayed, the active fractions can be chromatographed again on another

PGP-SP LC/MS system. In this system the C18-SP is be replaced by a cyanopropyl-SP. The use of a second SP with different molecular interactions will increase the possibility that peaks that co-eluted on the C18-SP will be separated in the next screen.

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Alternative approaches include other chromatographic phases such as the aminopropyl or phenyl-bonded SPs. In addition, an immobilized HSA-SP can also be employed in order to eliminate compounds that display extensive protein binding and, therefore, limited bioavailability. The fractions can be collected as described above and assayed in accord with the teachings herein.

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# Steps 1 and 2 - Throughput:

Once in operation, it is expected that the Initial Chromatographic Screen should take approximately four hours per extract and the Secondary Chromatographic Screen of the primary fractions an additional 4 hours for a total of eight hours and three extracts could be processed per day. This assumes that only one Initial Chromatographic System is utilized and the systems are run sequentially. If a second Initial Chromatographic System is brought on-line, the throughput should be about 6 extracts/day and 1000 extracts/year. Of course, these throughput conditions are exemplary and can be varied by one skilled in the art, as desired.

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#### **EXAMPLE 3**:

# Preparation of mg or greater quantities of active lead compounds:

A flow-chart for the isolation of the active compounds can be developed from the chromatographic experiments. The biological extracts containing these compounds can be processed using a flow-chart. For illustrative purposes, a sample flow chart is presented:

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- A. Initial Chromatographic Screen: Take the 3rd fraction after the VBL retention volume.
- B. Secondary Chromatographic Screen: Inject the fraction obtained from step A and collect the fraction eluting at 25 minutes.
- 5 C. Third Chromatographic Step: Inject the fraction obtained from Step B on the chromatographic system and collect the fraction eluting at 20 minutes.
  - D. Fourth Chromatographic Step, etc., until homogeneity is obtained.

If required, multiple columns can be used is the Initial Chromatographic Screen and larger format columns, i.e., a standard 150 mm x 4.1 mm ID column or greater, can be employed in the Second Chromatographic Screen. Since the initial process has the capacity to process kg quantities of botanical extracts, mg and greater quantities of active lead candidates can be prepared. Thus, enough compound can be isolated to fully characterize the molecular structure of an active lead compound and to fully characterize the *in vitro* activity of this compound. If the compound proves promising, larger quantities could also be produced for preliminary *in vivo* studies if an adequate chemical synthesis has not been developed using methods of chromatographic isolation of the active compounds from complex biological matrices known in the art.

In view of the above the PGP-SP can be used to characterize ligandbiopolymer interactions, including the direct determination of binding affinities; the identification of specific sites at which a ligand binds; elucidation of ligandligand binding interactions including competitive and allosteric interactions.

Using the method described herein, it is possible to undertake studies with lead drug candidates isolated in the screening program and known PGP substrates in order to identify PGP binding sites and ligand-ligand binding

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interactions and to create quantitative structure-binding relationships for directed combinatorial synthesis.

### EXAMPLE 4:

# Determination of PGP-substrate - Drug Candidate Binding Interactions on PGP

# a. Experimental Approach:

Both frontal and zonal LC techniques can be used in these studies. In general, the technique followed is outlined as: 1) The marker PGP-substrates [3H]-VBL, [14C]-ADR and [3H]-TAX is be used as the "solutes" and they are injected individually onto the PGP-SP column and their k's measured; 2) the drug candidates isolated and identified in the chromatographic screening process are the "displacers". Each "displacer" is added individually to the mobile phase and its concentration is systematically increased during a series of experiments; 3) the effect of the "displacer" concentrations on the k's of the "solutes" can then be measured and the Kd's of VBL, ADR and TAX established as well as the independence or overlap of their respective binding sites. Indeed, initial PGP-SP studies of the Kd of VBL have determined a Kd of 19±20 nM.

# b. Analysis of results:

When the "solute" is chromato-graphed using a mobile phase that does not contain the "displacer", k' is directly proportional to its binding affinity to PGP. When the "displacer" is added to the mobile phase, the magnitude and direction of the resulting changes in k' can be used to determine if the "displacer" binds at the same site(s) as the marker PGP-substrates and to indicate if co-operative {k' increases}, anti-co-operative {k' decreases} and non-co-operative {k' decrease} interactions occur between the "solute" and "displacer". The relationship

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between the k' of the solute and the mobile phase concentration of the displacer is expressed by the equation 1:

$$\frac{1}{(k'-x)} = \frac{VMK2[D]}{K3mL} + \frac{VM}{K3mL}$$
 Eqn 1

Where: VM = void volume of the column; K2 and K3 = equilibrium constants for the binding of the displacer and solute, respectively; mL = moles of the solute bound to the stationary phase; [D] = concentration of the displacer in the mobile phase; X = residual k' resulting from binding at sites unaffected by the displacer.

The term X is a constant that represents the portion of k' resulting from the binding of the solute to sites at which the displacer does not compete. If both the solute and displacer bind at only one identical site on PGP, then X = 0. Eqn. 1 predicts that when X = 0 a plot of 1/(kl) versus [D] will produce a linear relationship with a slope of (VMK2/K3mL) and an intercept of (VM/K3mL). The value of K2, the binding affinity constant for the displacer, can be determined directly by calculating the ratio of the slope to intercept for this plot. The inverse of the slope gives mL/Vm, which is the effective concentration of the binding sites in the column.

#### **EXAMPLE 5**:

Development of Quantitative Structure-Retention Relationships {OSRR}

# a. Approach:

The development of the QSRR analysis of the data set can be run according to the procedures previously published. In brief, the lead PGP-drugs can be chromatographed on the PGP-SP under the same experimental conditions and their k's determined. The molecular structures of the compounds will also

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be constructed using InsiteII 95.0 running on a Silicon Graphics Indy workstation and the molecular descriptors of the structures calculated {i.e., hydrophobicity, molecular volume, electronic distribution, molecular geometries, etc.} using Tsar V2.41, Mopac V6 and InsiteII 95.0 software and the Connolly surfaces will be calculated using MOLCAD {Tripos} software all running on the same workstation.

#### b. Analysis of data:

The k's of the lead PGP-drugs can be correlated to their molecular descriptors using the multivariate regression analysis program in Tsar V2.41. The simplest possible relationship between the descriptors can be established and the predictive power of the model determined by "leave-one-out" cross-validation.

# c. Pharmacophore Modeling:

The pharmacophores can be developed as previously described in the art. In brief, pharmacophores can be built using Apex-3D 95.0 software run on the Silicon Graphic Indy workstation. The pharmacophores can be constructed using all of the compounds in the set with a match superimposition greater than 0.7. 3D-QSAR equations can be derived with the site radius initially set at 1.3, the occupancy at 5, the sensitivity at 2.5 and the randomization at 500.

The data from the chromatographic, QSAR and pharmacophore modeling can provide direction to the combinatorial synthesis of new compounds to bring effective therapeutic agents to clinical trial. In addition, the knowledge gained using methods of the invention can be used to produce additional stationary phases with different biochemical targets such as Protein Kinase C. Thus, the screening of extracts and combinatorial pools can be expanded to other therapeutic areas.

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Using methods of the invention, the supports with many different moieties that bind to ligands may be exposed to drugs or inhibitors, then to drugs followed by chromatographic evaluation of the presence of the drug by chromatographic means to determine whether the drug is present on the support. Using means of the invention, it is also possible to determine whether proposed inhibitors of interaction will, in fact, prevent an interaction by exposing the prepared support having the appropriate protein bound thereto to proposed inhibitors, then to the toxin or drug followed by chromatographic evaluation of the support to determine whether the toxin or drug has been prevented from binding by the inhibitor under consideration.

While the invention has been exemplified using PCP, any ligand system may be used. Supports such as hydrophilic verticle support systems may be used in the methods of the invention.

An alternative experimental approach to the determination of binding affinities is affinity chromatography. We have previously reported the synthesis of a liquid chromatographic stationary phase containing immobilized PGP and its use in the determination of PGP binding affinities.

The following examples provide further characterization of the PGP-stationary phase and disclose the use of frontal and zonal chromatographic techniques to investigate the binding of vinblastine, doxorubicin, verapamil and cyclosporin A to the immobilized PGP. The compounds were added individually to the chromatographic system with or without ATP on the running buffer. The compounds were also added in pairs using standard competitive chromatography procedures. The results of the study demonstrate that both competitive and allosteric interactions occurred during the chromatographic studies and indicate that the immobilized PGP retained its conformational mobility.

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The following materials and methods were used in these additional Examples.

### **MATERIALS AND METHODS**

Materials: Immobilized Artificial Membrane (IAM). PC particles were obtained from Regis Chemical Co (Morton Grove, IL, USA). Glass column (HR5/5) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). [3H] vinblastine and [3H]cyclosporine A were purchased from Amersham Life Science Products (Boston, MA, USA). [3H]verapamil was from NENTM Life Science Products, Inc (Boston, MA, USA). Vinblastine, verapamil, doxorubicin, cyclosporin, CHAPS, glycerol, benzamidine, albumin bovine, were from Sigma Chemical Co. (St. Louis, MO, USA). GF/C glass microfiber filters were from Whatman. Scintillation liquid (Flo-Scint V) was purchased from Packard Instruments (Meriden, CT, USA).

Preparation of membranes: As previously described, the PGP-positive MDA435/LCC6<sup>MDR1</sup> cell line was obtained by transduction of PGP-negative expressing MDA435/LCC6 human breast cancer cells with a retroviral vector carrying MDR1 cDNA {PGP} (30). In these studies, about 80 x 10<sup>6</sup> cells were harvested in 10 ml of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 μM Leupeptin, 2 μM phenylmethanesulfonyl fluoride and 4 μM pepstatin). The suspension of cells was homogenized for 2 x 30 s (with a cooling period inbetween) with a Brinkmann Polytron homogenizer. The homogenized membrane was centrifuged first at 1,000 x g for 10 min, the pellets were discarded and the supernatant was collected and centrifuged at 150,000 x g for 30 min again. The membrane pellets were collected.

Immobilization of PGP on IAM particles: The membrane pellets were resuspended in 6 ml solubilization solution (50 mM Tris-HCl, pH 7,4, 500 mM

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NaCl, 15 mM CHAPS, 2 mM DTT, 10 % glycerol) for 3 hours at 4°C. Then this was mixed with 100 mg of dried IAM PC particles, and stirred for 1 hour at room temperature. The suspension of PGP-IAM was then dialyzed against dialysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM benzamidine) for 36 hours at 4°C (1.5 L for every 12 hours).

Preparation of liquid chromatographic column: The IAM PC particles with immobilized PGP were packed into a HR5/5 glass column (0.5 x 0.8 cm) after 3 times centrifugation at 350 x g, 3 min, at 4°C. Then the column was equilibrated with buffer B (50 mM Tris-HCl, pH 7.4) at room temperature for 3 hours.

Frontal chromatographic studies: The chromatographic system has been previously described (29) and was primarily based upon the PGP-IAM column connected on-line to a flow scintillation monitor (Radiometric FLO-ONE® Beta 500 TR instrument, Packard Instruments). All chromatographic experiments were conducted at room temperature using a flow rate of 0.5 ml/min.

The marker ligand, either [³H]-vinblastine [[³H]-VBL, 1.0 nM], [³H]-verapamil [[³H]-VER, 0.3 nM] or [³H]cyclosporine A [[³H]-CsA, 2.0 nM] were applied to the PGP-IAM column in sample volumes of 25-50 ml. The solutions containing the marker ligands were supplemented with a range concentrations of either cold VBL, VER, doxorubicin or CsA. Elution profiles were obtained showing front and plateau regions as illustrated for [³H]VER in Figure 2. The observed elution volume data were used for calculation of ligand dissociation constants. The K<sub>d</sub> values of VER and CsA were calculated by nonlinear regression, as illustrated for [³H]-VER Fig. 3, using Prism (GraphPad Software) and a one-site binding (hyperbola), equation (2) below:

$$Y = Bmax \cdot X / (kd + X)$$
 (2)

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In which: X is the concentration of VER or CsA; Y is equal to [verapamil] (V –  $V_{min}$ ) or [CsA](V –  $V_{min}$ ), where:  $V_{min}$  is the elution volume of VER or CsA under conditions where specific interactions are completely suppressed and V is the retention volume of VER or CsA at different concentrations (0.3 – 400  $\mu$ M for VER and 2.5 – 100 nM for CsA).

Two series of runs were made to determine the  $K_d$  value for VBL and the  $K_1$  values for doxorubicin and CsA. One series was performed with different concentrations of cold VBL (3 nM - 100 nM) to displace [ $^3$ H]-VBL, and the other was performed with different concentrations of cold doxorubicin (5  $\mu$ M - 70  $\mu$ M) or CsA (10 nM - 250 nM) with [ $^3$ H]-VBL as the displaced ligand. The  $K_d$  value of VBL and the  $K_1$  values of doxorubicin and CsA were calculated using equations (3) and (4) below.

$$(V \max - V)^{-1} = (1 + [VBL]K_{VBL}) \cdot (V \min[P]K_{VBL})^{-1} + (1 + [VBL]K_{VBL})^{2} \cdot (V \min[P]K_{VBL} K_{i})^{-1} \cdot [I]^{-1}$$
(3)

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$$(V-V_{min})-1 = (V_{min}[P]K_{VBL})^{-1} + (V_{min}[P])^{-1}[VBL]$$
 (4)

Where: I represents doxorubicin or CsA; [P] the concentration of active receptor in the volume;  $V_{min}$ , the elution volume of VBL under conditions where the specific interaction is completely suppressed;  $V_{max}$  is the elution volume obtained with 1.0 nM [ $^3$ H]VBL.

Control Experiments: Membranes from the PGP-negative parental cell line, MDA436/LCC6 (30), were prepared and immobilized on an IAM support as described above. Using the procedure described above, the PGP-negative-IAM support was packed into a glass column (0.5 x 0.8 cm) and a second glass column (0.5 x 0.8 cm) was packed with untreated IAM support. The three columns, IAM support {negative control} PGP-negative-IAM {positive control}

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and PGP-IAM {experimental} were separately connected on-line to a flow scintillation monitor and used in zonal chromatographic experiments. In these studies, a mobile phase composed of Tris-HCl [50 mM, pH 7.4] was constantly pumped through the column at a flow rate of 0.5 ml/min. A single 100 ml injection of the marker ligand, [3H]VER [23.5 nM], was injected onto the column and the radioactive signal (CPM) was recorded every six seconds. The chromatographic data was evaluated in 0.5 minute intervals and smoothed using the Microsoft Excel program with a 5-point moving average.

Membrane Binding Assays: The binding assays were accomplished using a previously described method. Briefly, 50 μl of [³H]VBL [3 nM – 100 nM with 2 % ethanol (v/v) was incubated with PGP-containing or PGP-negative membranes (150 μg in 50 μl) or bare IAM particles and 50 μl of cold VBL [12 μM] for two hours at room temperature. Bound and free drug were separated by rapid filtration through Whatman GF/C filters which had beed pre-soaked with 0.1% BAS inTris-HCl [50 mM, pH 7.4]. The filters were then washed with two portions of 5-ml ice-cold 20 mM Tris-HCl, 20 mM MgCl<sub>2</sub> buffer. The filters were dried, and retained radioactivity was quantitated by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding

Protein Assay: The amount of membrane and the immobilized membrane were determined by bicinchoninic acid (BAC) protein assay. The sample was diluted with NaOH [0.1 M]. A protein standard (0.3 – 37.5 μg in 50 μl) was prepared with Albumin standard (Pierce, Rockford, IL). The measurement procedure followed the instruction in Pierce BCA protein assay kit in which 20 ml of reagent A was mixed with 0.4 ml of reagent B. Aliquots [50 μl] of standards and samples were added in triplicate to a 96-well plate and 200 μl of

BCA reagent (A+B) was added in each well. The standards and samples were incubated at room temperature for three hours and the resulting absorbance at 1 = 570 nm was determined using a spectrophotometer. The amount of protein was calculated by using the Microsoft Excel program.

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# **EXAMPLE 6**:

Chromatographic studies with vinblastine and doxorubicin

The dissociation constants (K<sub>d</sub>) of vinblastine (VBL) and doxorubicin were determined on the PGP-IAM stationary phase using displacement chromatography with [<sup>3</sup>H]VBL as the marker ligand, Table 1 below.

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TABLE 1

The  $k_d$  values calculated using frontal affinity chromatography on the

| <del></del> | Drugs                      | $k_d^{\ a}$  | k <sub>d</sub>  |
|-------------|----------------------------|--|---|
|             | Vinblastine                | $23.5 \pm 7.8$ nM                                    | $37.0 \pm 10$ nM <sup>b</sup><br>$36.0 \pm 5$ nM <sup>c</sup>   |
|             | Verapamil                  | $54.2 \pm 4.6 \mu M$                                 | $0.45 \pm 0.05 \text{ mM}^{\text{b}}$   |
|             | Doxorubicin Cyclosporine A | $15.0 \pm 3.2 \mu M^d$<br>$62.5 \pm 5.6 \text{ nMe}$ | $31 \pm 7.3 \text{ mM}^{\text{b}}$<br>$18 \pm 3.6 \text{ nM}^{\text{b}}$<br>$97.9 \pm 19.4 \text{ nM}^{\text{d}}$ |

- a. These values were measured in the present work
- b. These values are from literature values.

immobilized PGP-IAM stationary phase

- c. It is from the literature value.
- d. The values were obtained by displacing [3H]vinblastine (see methods).

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e. It was measured when 3 mM ATP was in the running buffer.

The calculated  $K_d$  of VBL was 23.5 ± 7.8 nM which is consistent with the previously reported values of 37.0 ± 10 nM (18) and 36 ± 5 nM. The  $K_d$  value of 15.0 ± 3.2  $\mu$ M determined for doxorubicin was also consistent with the reported value of 31.0 ± 7.3  $\mu$ M.

The chromatographic results were also consistent with the results obtained from binding assays using the same membranes utilized in the construction of the PGP-IAM stationary phase. In these studies, membrane extracts were prepared from the PGP-expressing cell line, MDA435/LCC6<sup>MDR1</sup>, and the PGP-negative cell line, MDA435/LCC6 (30). VBL binding to the two membrane extracts and the IAM support was determined using a previously described rapid filtration method. No specific binding was observed with the PGP-negative cell membranes or the IAM particles, while a  $K_d$  value of 54.5  $\pm$  40.8 nM was determined using the membranes from the PGP-expressing cell line. The calculated affinity was consistent with the previously published value,  $37 \pm 10$  nM, obtained using the same experimental approach. In these experiments, it was necessary to add ethanol (2%, v/v) to the [ $^3$ H]VBL solution to prevent non-specific binding to the walls of the polypropylene tubes.

# EXAMPLE 7:

20 Chromatographic studies with verapamil and vinblastine:

When verapamil (VER) was used as the displacer of the [ $^3$ H]VBL marker ligand, the calculated  $K_d$  value for VER was 54.2  $\pm$  4.6  $\mu$ M. This value was significantly higher than the previously reported values of 0.45  $\pm$  0.05  $\mu$ M, and 0.6  $\pm$  0.18  $\mu$ M. When the experimental conditions were reversed and [ $^3$ H]VER

was the marker ligand and VBL the displacer, no displacement of [<sup>3</sup>H]VER was observed when 50 nM and 100 nM concentrations of VBL were added to the mobile phase, Table 2 below.

### TABLE 2

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Retention volumes of [<sup>3</sup>H]vinblastine and [<sup>3</sup>H]cyclosporine A obtained when (1) no ATP was present in the running buffer, (2) 3 mM ATP was added in the running buffer, (3) 50 nM cold vinblastine supplemented in the sample (no ATP in the buffer), and (4) 100 nM cold vinblastine was in the sample (no ATP in the buffer).

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Retention volume (ml) at

| vinblastine                   | No ATP | 3 mM ATP | 50 nM vinblastine       | 100 nM  |
|-------------------------------|--------|----------|-------------------------|---------|
| Viiioiastine                  |        |          | (no ATP)                | (noATP) |
| [³H]Vinblastine               | 32.1   | 8.4      | 11.0                    | 9.5     |
| [3H]verapamil                 | 34.2   | 5.9      | 34.1                    | 34.0    |
| [ <sup>3</sup> H]Cyclosporine | A 7.8  | 17.5     | 15.7(15.4) <sup>a</sup> | 18.8    |

a.15.7 ml was measured at the condition of no ATP was present in the running buffer, and 15.4 ml was obtained when 3 mM ATP was in the running bufer.

The specificity of the chromatographic interactions of VER with the immobilized PGP were investigated through the independent immobilization of membrane extracts from the PGP-expressing cell line and the PGP-negative cell line on the IAM-support. Zonal chromatographic studies were conducted with columns containing either the PGP-IAM, PGP-negative-IAM or IAM support.

When a 100 ml sample of [3H]VER was injected onto the columns containing

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either the PGP-negative-IAM support or the IAM support itself, the retention volumes were the same, Figure 4, curves 1 and 2. This indicates that there was no specific interactions with the immobilized membrane extracts obtained from the PGP-negative cells relative to the non-specific interaction that occur between [<sup>3</sup>H]VER and the IAM support. On the column containing the PGP-IAM support, the retention volume of [<sup>3</sup>H]VER was > 20 ml, Figure 4, curve 3. This indicates that specific binding interactions occurred between the [<sup>3</sup>H]VER and the immobilized membrane extracts obtained from the PGP-expressing cells.

### **EXAMPLE 8:**

10 Chromatographic studies with cyclosporin A and vinblastine:

When cyclosporin A (CsA) was used as the displacer of the [³H]VBL marker ligand, the calculated K<sub>d</sub> value for CsA was 97.9 ± 19.4 nM as compared to the previously reported value of 18.0 ± 3.6 nM, Table 1. When [³H]CsA was used as the marker ligand and migrated alone through the PGP-IAM, the retention volume was 7.8 ml, Table 2, and no specific retention was observed, Fig. 5A. The addition of 50 nM VBL to the running buffer increased the retention volume of [³H]CsA to 15.7 ml, Table 2, and produced the expected frontal chromatogram, Fig. 5B. When the VBL concentration was increased to 100 nM, the observed retention of the frontal chromatogram increased to 18.8 ml, Fig. 5D,Table 2.

#### EXAMPLE 9:

Effect of ATP on the chromatographic properties of the PGP-IAM:

The addition of 3 mM ATP to the running buffer resulted in changes in the retentions of CsA, VBL and VER. In the case of CsA, the addition of ATP increased the retention volume from 7.8 ml to 17.5 ml, Table 2, and the observed chromatogram changed from non-specific elution, Fig. 5.A, to a frontal

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chromatogram displaying specific retention to the immobilized PGP-IAM, Figure 4. With 3mM ATP in the running buffer, [<sup>3</sup>H]CsA was displaced from PGP by the addition of unlabeled-CsA. The results fom the CsA displacement studies were used to calculate a K<sub>d</sub> value of 62.5 nM for CsA binding to the immobilized PGP.

When VBL was the marker ligand, the addition of 3 mM ATP decreased the retention volume from 32.1 ml to 8.4 ml, Table 2. The presence of ATP in the running buffer also changed the observed chromatograms from a frontal curve demonstrating specific retention, Figure 6A, to a non-specific curve, Figure 6B. A similar effect was observed for VER as the addition of 3 mM ATP to the running buffer reduced the elution volume from 34.2 ml to 5.9 ml, Table 2, with a resulting loss in specific retention, as demonstrated by the shape of the frontal curve (data not shown).

### **CONCLUSIONS**

The synthesis and characterization of a novel liquid chromatographic

stationary phase containing immobilized P-glycoprotein (PGP-IAM) is described supra. The stationary phase was prepared using solubilized membranes from PGP-expressing cells. The donor cells were MDA435/LCC6<sup>MDR1</sup>, a cell line obtained by retroviral transduction of MDR1 cDNA (coding for PGP) into

MDA435/LCC6 human breast cancer cells. In this study, a second liquid chromatographic stationary phase was prepared through the immobilization of membrane extracts from the PGP-negative MDA435/LCC6 parental cell line.

A comparison of the chromatographic retention of verapamil, a known PGP substrate, on the native chromatographic support and the PGP-positive and PGP-

negative supports, Figure 5 demonstrated that, for PGP substrates, the observed

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chromatographic retentions were a function of specific interactions between the substrate and the immobilized PGP.

The relationship between chromatographic retention on the PGP-IAM stationary phase and PGP binding affinity was also illustrated by the comparison of the substrate affinities calculated from the chromatographic results obtained using the PGP-IAM column with the results from classical filtration binding assays, Table 1. The initial studies in this series were conducted using [<sup>3</sup>H]-vinblastine ([<sup>3</sup>H]VBL) as the marker ligand and Tris buffer [50 mM, pH 7.4] as the running buffer. Under these conditions cyclosporin A (CsA) displaced [<sup>3</sup>H]VBL producing a calculated K<sub>d</sub> value of 97.9 nM, Table 1, which is consistent with the results of previous studies based on filtration binding assays.

The displacement of [<sup>3</sup>H]VBL by CsA indicated that CsA specifically and competitively bound to immobilized PGP. However, when [<sup>3</sup>H]CsA was used as the marker ligand, frontal chromatography with [<sup>3</sup>H]CsA alone in the running buffer produced a low retention volume, 7.8 ml (Table 2) and no detectable specific retention, see Figure 5A. This indicated that under the experimental conditions, [<sup>3</sup>H]CsA did not specifically bind to immobilized PGP.

The contradiction between the data obtained with [3H]VBL as the marker versus the data obtained with [3H]CsA alone in the running buffer was eliminated when VBL was added to the running buffer. The addition of 50 nM VBL to the running buffer produced a classical frontal chromatogram for [3H]CsA (Figure 5B) and increased the retention volume to 15.7 ml, Table 2. When the VBL concentration was increased to 100 nM, the retention volume also increased to 18.8 ml, Table 2, Figure 5D.

The results from the studies with [3H]VBL and [3H]CsA as the marker ligands indicate that the addition of VBL to the running buffer produces a co-

operative allosteric interaction in the binding process between [<sup>3</sup>H]CsA and the immobilized PGP. This suggests that the immobilized PGP has retained its conformational mobility and that the binding of VBL to PGP opened up the site at which CsA binds.

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The data also indicates that once the VBL-induced conformational change has occurred, CsA can bind to PGP and displace VBL through competitive and/or anti-cooperative allosteric interactions. The addition of CsA to the running buffer did not change the shape of the [³H]VBL frontal chromatograms demonstrating that the displacement was competitive in nature. One explanation for these results is that the VBL induced CsA binding site is contiguous with or part of the VBL site. CsA binding to the induced site on PGP does not directly compete with VBL for the same site, but inhibits VBL binding through steric interactions. Korzekawa, et al. have proposed a similar model for enzymatic inhibition as well as activation in some isoforms of cytochrome P450. In this model, the simultaneous but independent binding of two different substrates in the active site of the enzyme results in steric interactions that produce the displacement {inhibition} or re-orientation {activation} of one of the substrates.

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In these studies, the addition of increasing concentrations of VER to the running buffer reduced the retention volumes of [³H]VBL without changing the shapes of the frontal chromatograms. This indicates that VER competitively displaced VBL from its binding to PGP, although the calculated K<sub>d</sub> value was significantly higher than previously reported values, Table 1. However, VBL was unable to displace [³H]VER from the immobilized PGP. These results suggest that VER binds to two or more distinct sites on the PGP molecule including the site at which VBL binds. Furthermore, the site that is common to VBL and VER is not the primary VER binding site, i.e., the site for which VER

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has the highest affinity. Thus, the Kd value calculated from the frontal chromatographic studies, Table 1, appears to be the sum of VER affinity to the different VER binding sites. The experimental conditions used in this study could not determine if the VER and VBL sites are allosterically linked. Further studies will be effected to select specific markers for the high and low affinity VER binding sites.

The existence of multiple binding sites on the PGP molecule has been suggested by the results of several previous studies. Using classical filtration binding assays, Ferry et al. Obtained evidence of non-overlapping binding sites for Vinca alkaloids and dihydropyridine substrates, and perhaps also for Vinca alkaloids and doxorubicin. Also, distinct sites for steroids and Vinca alkaloids, steroids and VER, VER and dihydropyridines, and between different steroids, were supported by the results of studies using an ATPase activation endpoint. Moreover, separate binding sites have been suggested for VER and anthracyclines, VER and colchicine, and cyclosporins and dihydropyridines.

PGP is a member of the ATP-binding cassette superfamily and ATPase activity plays a role in substrate transport. Thus, it should be expected that the addition of ATP to the running buffer would change the chromatographic properties of the immobilized PGP chromatographic system. In this study, the addition of 3 mM ATP to the running buffer increased the retention volume of [<sup>3</sup>H]CsA from 7.8 ml to 17.5 ml (Table 2), produced a classical frontal chromatogram for [<sup>3</sup>H]CsA (Figure 5C) and permitted the calculation of an a k<sub>d</sub> value of 62.5 nM, Table 1. These results indicate that the addition of ATP to the running buffer produced a cooperative allosteric interaction that increased the binding affinity of PGP for CsA.

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The presence of ATP in the running buffer produced the opposite effect on the retention volumes of [3H]VBL and [3H]VER. When [3H]VBL was the marker ligand, the addition of 3 mM ATP reduced the observed retention from 32.1 ml to 8.4 ml, Table 2.. The reduction in [3H]VBL retention volume was accompanied by an apparent loss of specific retention, Figure 4, suggesting an anti-cooperative allosteric interaction. This possibility is supported by the results of VBL displacement experiments. The addition of unlabeled VBL to the running buffer, in the absence of 3 mM ATP also decreased the retention volume of [3H]VBL, Table 2. However, the effect of VBL on [3H]VBL retention differs from the effect observed with ATP as specific frontal chromatographic curves were observed after the addition of 50 nM and 100 nM VBL {data not shown}. This indicates that the decreased retention was due to competitive displacement of [3H]VBL by VBL. The ATP induced reduction in the retention volume for [3H]VER, 34.2 ml to 5.9 ml, coupled with the loss of specific retention, also indicate that the primary site of VER binding to the PGP molecule was affected by an anti-cooperative allosteric interaction.

These results support the observations from the VBL-CsA binding interaction studies that the immobilized PGP has retained it conformational mobility. Thus, the addition of ATP to the running buffer produced a conformational change in the immobilized PGP molecule that opened up the site at which CsA binds. The same conformational change that increased the binding affinity for CsA also altered the site at which VBL binds, decreasing the affinity of PGP for VBL. The effect of VBL on CsA binding affinity and the effect of ATP on the binding affinities of VBL and CsA indicates that separate, but closely linked, binding sites for CsA and VBL exist on the PGP molecule.

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The observation of ATP-induced conformational shifts in the immobilized PGP molecule is consistent with results from previous studies. An infrared spectroscopy study has shown that PGP changes conformation a first time when ATP is added, and then again when a substrate is added in the presence of ATP. The changes in affinity for VBL and CsA that we observed following the addition of ATP most probably reflects the second of the above conformational changes, or the situation that immediately follows substrate binding and ATPase activation.

To our knowledge, the results reported herein, and specifically the dramatic effect of VBL on CsA binding to PGP, are the first to strongly suggest distinct and allosterically connected sites for Vinca alkaloids (VBL) and cyclosporins. The results of previous studies of dissociation rates were consistent with overlapping binding sites for CsA and VBL.

The mechanism by which PGP causes the efflux of several substrates has been discussed for many years and remains incompletely defined. In the most widely accepted model, PGP is proposed to work as an "hydrophobic vacuum cleaner", binding its substrates from the inner leaflet of cell membrane and transporting them to the extracellular space, or perhaps "flipping" them to the outer leaflet. Both transport mechanisms assume a conformationally mobile molecule capable of responding to ATPase activity and substrate/inhibitor binding.

The immobilized PGP liquid chromatographic stationary phase described herein appears to reproduce PGP substrate binding as determined by classical filtration binding assays. Observed binding in this system is PGP-specific and highly sensitive to conformational changes caused by PGP interactions with substrates and ATP, reflecting changes occurring in the functional cycle of

PGPGP Thus, PGP-affinity chromatography represents a promising tool for a quick and reproducible evaluation of potential PGP substrates and/or inhibitors and provides a useful probe of the transport mechanism.

### WHAT IS CLAIMED IS:

- 1. A method for identifying, isolating and/or characterizing ligands that interact with p-glycoprotein comprising:
- (i) immobilizing p-glycoprotein on an artificial membrane support which is on-line with a liquid chromatography system; and
- (ii) continuously contacting said immobilized P-glycoprotein with a liquid chromatographic system that contains one or more ligands that are to be identified, isolated and/or characterized based on their specific interaction with said immobilized P-glycoprotein.
- 10 2. The method of Claim 1 which further comprises eluting ligands that bind to P-glycoprotein on said artificial membrane support.
  - 3. The method of Claim 1, which further comprises evaluating the elution profile of said ligands.
- 4. The method of Claim 1, wherein an identified ligand is evaluated to determine its effect on breast cancer cells *in vitro*.
  - 5. The method of Claim 1, wherein an identified ligand is evaluated in an animal xenograft model of human breast cancer.
  - 6. The method of Claim 1, wherein the immobilized PGP is complexed with a compound that specifically binds PGP and the method is used

to continuously identify ligands that specifically bind PGP by a displacement competition binding assay.

- 7. The method of Claim 1, wherein said identified ligands are further characterized by passing an eluate containing said ligand through another testing device.
- 8. The method of Claim 7, wherein said device is a mass spectrometer.
- 9. The method of Claim 1, which is used to identify compounds for treating MDR1/PGP resistant tumors.
- 10. The method of Claim 1 which comprises more than one chromatographic screen using columns comprising different P-glycoprotein-containing compounds or derivatives.

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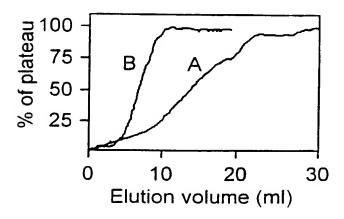


FIG. 1

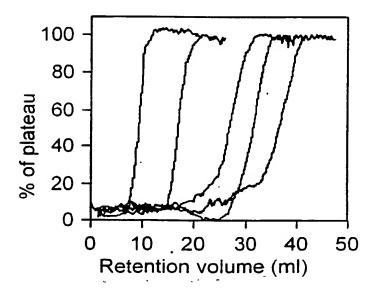


FIG. 2

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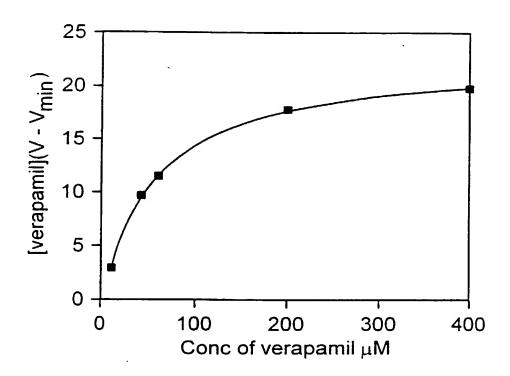


FIG. 3

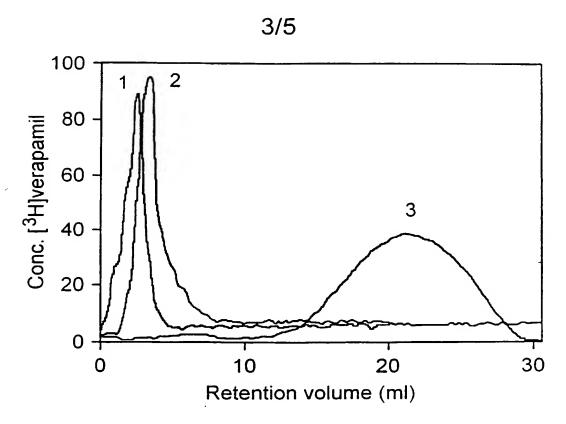


FIG. 4

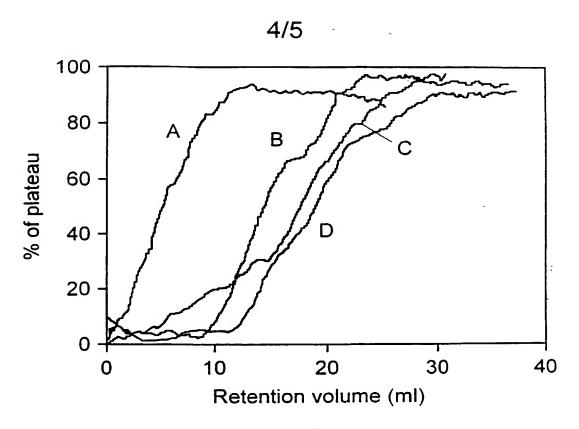


FIG. 5

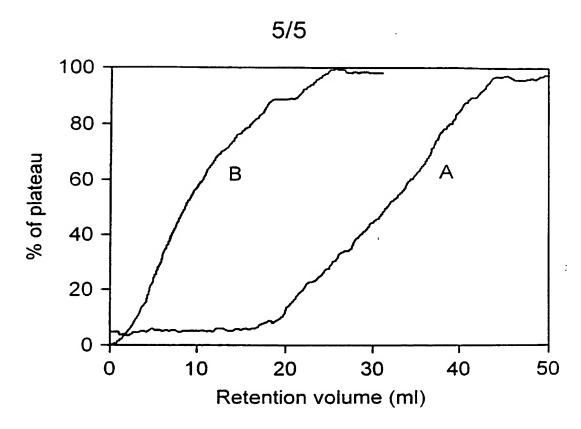


FIG. 6

Yes

No

Page 1

Docket No.: 1908-006-27

#### Declaration, Power of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

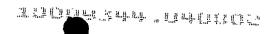
We (1) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

#### CHROMATOGRAPHIC DETERMINATION OF P-GLYCOPROTEIN REACTIVE LIGANDS the specification of which is attached hereto. was filed as Application Serial No. 10/009,344 and amended on was filed as PCT international application PCT/US00/15820 Number OR June 9, 2000 and was amended under PCT Article 19 (if applicable). on We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations. We (I) hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s) Application No. Country Day/Month/Year Priority Claimed Yes No Yes No 25Y No

We (I) hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

|  | 60/151,402  | June 9, 1999   |  |   |  |
|--|---|--|--|---|--|
| (A   | (Application Number)  (Application Number)  |  | (Filing Date)  |   |  |
| (A   |   |  | (Filing Date)  |   |  |
| International application<br>claims of this application<br>provided by the first par<br>patentability as defined   | n designating the United Sta<br>on is not disclosed in the pr<br>agraph of 35 U.S.C. §112, I  | ates, listed below and,<br>rior United States or I<br>acknowledge the duty<br>me available between t   | insofar as to<br>CT Internati<br>to disclose i   | cation(s), or §365(c) of any Potential in the man information which is material cofficient and cofficient and coffice the prior application and   |  |
| Application Serial N   | lo. Filing D  | rate Statu   | s (pending, j  | patented, abandoned)  |  |
| Kimball, Reg. No. 34,64<br>Reg. No. 43,804; Lisa K<br>Reg. No. 47,258, as our<br>and to transact all busine  | 11; Wilburn L. Chesser, Reg.<br>Norton, Reg. No. 44,977; P<br>r (my) attorneys, with full p<br>ess in the Patent Office conn  | g. No. 41,668; James Marrick R. Delaney, Regowers of substitution a nected therewith; and y  | 4. Heintz, R<br>z. No. 45,338<br>and revocation<br>ve (I) hereby   | eider, Reg. No. 24,765; Paul<br>eg. No. 41,828; Amy L. Mill<br>is and Christopher W. Raimw<br>on, to prosecute this applicate<br>request that all corresponder  |  |
| Kimball, Reg. No. 34,64 Reg. No. 43,804; Lisa K Reg. No. 47,258, as our and to transact all busine regarding this application 1200 Nineteenth Street,  We (I) declare the on information and believeliful false statements   | AI; Wilburn L. Chesser, Reg. Norton, Reg. No. 44,977; Per (my) attorneys, with full persons in the Patent Office control by the sent to Supervisor, Patent N.W., Washington, D.C. 20 that all statements made here are believed to be true; and the like so made are punished.                  | p. No. 41,668; James Matrick R. Delaney, Regowers of substitution a nected therewith; and vitent Prosecution Servi 1036-2412.  In of our (my) own knowld further that these states shable by fine or impris  | M. Heintz, R. 2. No. 45,338 and revocation of the control of the c | eg. No. 41,828; Amy L. Mill a and Christopher W. Raimwon, to prosecute this applicate request that all corresponder farbury Rudnick & Wolfe Literate and that all statements made made with the knowledge tooth, under Section 1001 of T.   |  |
| Kimball, Reg. No. 34,64 Reg. No. 43,804; Lisa K Reg. No. 47,258, as our and to transact all busine regarding this application 1200 Nineteenth Street,  We (I) declare the on information and believely willful false statements at 18 of the United States (   | AI; Wilburn L. Chesser, Reg. Norton, Reg. No. 44,977; Per (my) attorneys, with full persons in the Patent Office control by the sent to Supervisor, Patent N.W., Washington, D.C. 20 that all statements made here are believed to be true; and the like so made are punished.                  | p. No. 41,668; James Matrick R. Delaney, Regowers of substitution a nected therewith; and vitent Prosecution Servi 1036-2412.  In of our (my) own knowld further that these states shable by fine or impris  | M. Heintz, R. 2. No. 45,338 and revocation (I) hereby ces, Piper M. welcdge are thements were comment, or be pardize the version of the comment.   | eg. No. 41,828; Amy L. Mill and Christopher W. Raimwon, to prosecute this applicate request that all corresponder farbury Rudnick & Wolfe Little and that all statements made made with the knowledge tooth, under Section 1001 of Tivalidity of the application or a                                     |  |
| Kimball, Reg. No. 34,64 Reg. No. 43,804; Lisa K Reg. No. 47,258, as our and to transact all busine regarding this application 1200 Nineteenth Street,  We (I) declare the on information and believellful false statements   | AI; Wilburn L. Chesser, Reg. Norton, Reg. No. 44,977; Per (my) attorneys, with full persons in the Patent Office control by the sent to Supervisor, Patent N.W., Washington, D.C. 20 that all statements made here are believed to be true; and the like so made are punished.                  | p. No. 41,668; James Matrick R. Delaney, Regowers of substitution a nected therewith; and vitent Prosecution Servi 1036-2412.  In of our (my) own knowld further that these states shable by fine or impris  | M. Heintz, R. 2. No. 45,338 and revocation (I) hereby ces, Piper M. welcode are tements were conment, or by pardize the same and 3003 346  | eg. No. 41,828; Amy L. Mill and Christopher W. Raimwon, to prosecute this applicate request that all corresponder farbury Rudnick & Wolfe Little and that all statements made made with the knowledge toth, under Section 1001 of Totalidity of the application or a street, N.W.                         |  |
| Kimball, Reg. No. 34,64 Reg. No. 43,804; Lisa K Reg. No. 47,258, as our and to transact all busine regarding this application 1200 Nineteenth Street,  We (I) declare the on information and believely willful false statements at 18 of the United States Opatent issuing thereon.                                  | AI; Wilburn L. Chesser, Reg. Norton, Reg. No. 44,977; Per (my) attorneys, with full press in the Patent Office conton be sent to Supervisor, Pat N.W., Washington, D.C. 20 nat all statements made here is fare believed to be true; and the like so made are punished and that such willful fa | p. No. 41,668; James Matrick R. Delaney, Regowers of substitution a sected therewith; and watent Prosecution Servi 1036-2412.  In of our (my) own knows further that these statements may jeen   | M. Heintz, R. 2. No. 45,338 and revocation (I) hereby ces, Piper M. welcode are tements were conment, or by pardize the same and 3003 346  | eg. No. 41,828; Amy L. Mill and Christopher W. Raimwon, to prosecute this applicate request that all corresponder farbury Rudnick & Wolfe Little and that all statements made made with the knowledge tooth, under Section 1001 of Tivalidity of the application or a                                     |  |
| Kimball, Reg. No. 34,64 Reg. No. 43,804; Lisa K Reg. No. 47,258, as our and to transact all busine regarding this application 1200 Nineteenth Street,  We (I) declare the on information and believely willful false statements at 18 of the United States C patent issuing thereon.  Irving WAINER                  | AI; Wilburn L. Chesser, Reg. Norton, Reg. No. 44,977; Per (my) attorneys, with full pless in the Patent Office conton be sent to Supervisor, Pat N.W., Washington, D.C. 20 and all statements made here of are believed to be true; and the like so made are punished and that such willful fa  | p. No. 41,668; James Matrick R. Delaney, Regowers of substitution a sected therewith; and watent Prosecution Servi 1036-2412.  In of our (my) own knows further that these statements may jeen   | M. Heintz, R. S. No. 45,338 and revocation (I) hereby ces, Piper M. S. William of the ments were sonment, or the pardize the s. 3003 346 Washington  | eg. No. 41,828; Amy L. Mill and Christopher W. Raimwon, to prosecute this applicate request that all corresponder farbury Rudnick & Wolfe Little and that all statements made made with the knowledge toth, under Section 1001 of Totalidity of the application or a street, N.W.                         |  |
| Kimball, Reg. No. 34,64 Reg. No. 43,804; Lisa K Reg. No. 47,258, as our and to transact all busine regarding this application 1200 Nineteenth Street,  We (I) declare the on information and belief willful false statements at 18 of the United States C patent issuing thereon.  Irving WAINER  NAME OF FIRST INVE | AI; Wilburn L. Chesser, Reg. Norton, Reg. No. 44,977; Per (my) attorneys, with full pless in the Patent Office conton be sent to Supervisor, Pat N.W., Washington, D.C. 20 and all statements made here of are believed to be true; and the like so made are punished and that such willful fa  | y. No. 41,668; James Matrick R. Delaney, Regowers of substitution a nected therewith; and watent Prosecution Servi 1036-2412.  In of our (my) own know of further that these states shable by fine or imprishes statements may jee Residence:            | M. Heintz, R. 2. No. 45,338 and revocation (I) hereby ces, Piper M. welcage are to tements were comment, or be pardize the comment (I) washington (I) the comment (I) washington (I) the comment (I) the comment (I) the comment (I) the comment (I) washington (I) the comment (I) the commen | eg. No. 41,828; Amy L. Mill  and Christopher W. Raimus on, to prosecute this applicate request that all corresponder farbury Rudnick & Wolfe LI  rue and that all statements made made with the knowledge to both, under Section 1001 of To  validity of the application or a  Street, N.W.  on, DC 20008 |  |
| Kimball, Reg. No. 34,64 Reg. No. 43,804; Lisa K Reg. No. 47,258, as our and to transact all busine regarding this application 1200 Nineteenth Street,  We (I) declare the on information and belief willful false statements at 18 of the United States C patent issuing thereon.  Irving WAINER  NAME OF FIRST INVE | AI; Wilburn L. Chesser, Reg. Norton, Reg. No. 44,977; Per (my) attorneys, with full pless in the Patent Office conton be sent to Supervisor, Pat N.W., Washington, D.C. 20 and all statements made here of are believed to be true; and the like so made are punished and that such willful fa  | p. No. 41,668; James Marick R. Delaney, Regowers of substitution a nected therewith; and watent Prosecution Servi 10036-2412.  In of our (my) own knowd further that these states shable by fine or imprishes statements may jee Residence:  Citizen of: | M. Heintz, R. 2. No. 45,338 and revocation (I) hereby ces, Piper M. welcage are to tements were comment, or be pardize the comment (I) washington (I) the comment (I) washington (I) the comment (I) the comment (I) the comment (I) the comment (I) washington (I) the comment (I) the commen | eg. No. 41,828; Amy L. Mill l; and Christopher W. Raimwon, to prosecute this applicate request that all corresponder farbury Rudnick & Wolfe Literate and that all statements made made with the knowledge to both, under Section 1001 of Toyalidity of the application or a Street, N.W.  On, DC 20008   |  |

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| We (I) hereby claim t        | ie benefit under Title 35 | , United States Code, | §119(e) of any | United States p | movisional |
|------------------------------|---------------------------|-----------------------|----------------|-----------------|------------|
| epplication(s) listed below. |                           |                       |                |                 |            |

|   | 60/151,40  | 02  | June 9   | , 1999   |   |
|---|--|---|--|--|---|
| <del></del>   | (Application N   | lumber)   | (Filing  | Date)  |   |
|   | (Application N   | lumber)   | (Filing  | z Date)  |   |
| International applications of this app<br>provided by the fire<br>patentability as de   | ication designating<br>dication is not disc<br>est paragraph of 35<br>fined in 37 CFR §1   | the United States, list<br>closed in the prior United U.S.C. §112, I acknow   | ted below and,<br>ted States or P<br>viedge the duty<br>lable between t  | insofar as t<br>CT Internat<br>to disclose   | ication(s), or \$365(c) of any PCI<br>the subject matter of each of the<br>tional application in the manne<br>information which is material to<br>to of the prior application and the   |
| Application S   | erial No.  | Filing Date   | Status   | (pending,  | patented, abandoned)  |
| Kimball, Reg. No. Reg. No. 43,804; I Reg. No. 47,258, and to transact all regarding this application Nineteenth S  We (I) deconting the statem willful false statem | 34,641; Wilburn I lisa K. Norton, Reg as our (my) attorned business in the Pattlication be sent to street, N.W., Washidare that all stateaned belief are believed that all the sources and the like so tates Code and that | L. Chosser, Reg. No. 4 3. No. 44,977; Patrick F sys, with full powers of ent Office connected the Supervisor, Patent Pro ington, D.C. 20036-24 ents made herein of our d to be true; and furthe made are punishable b | 1,668; James M. Delaney, Reg<br>f substitution a<br>nerewith; and w<br>secution Service<br>12.<br>(my) own known that these state<br>y fine or imprise | 1. Heintz, R. No. 45,333 and revocation (I) hereby ses, Piper M wledge are to ements wer comment, or least | teider, Reg. No. 24,765; Paul C. Reg. No. 41,828; Amy L. Miller, 8; and Christopher W. Raimund, on, to prosecute this application y request that all correspondence farbury Rudnick & Wolfe LLP, true and that all statements made we made with the knowledge that both, under Section 1001 of Title validity of the application or any |
| Irving WAINER   |  |   | Residence:   | 3003 34 <sup>th</sup>  | Street, N.W.  |
| NAME OF FIRST   | INVENTOR   |   |  | Washingto  | on, DC 20008  |
|   | · , .  |   | Citizen of:  | United S   | tates of America  |
| Signature of Inven  | tor  |   | Post Office  | Address:   | Same As Above   |
| Date  |  |   |  |  |   |

Apr 8 2002 12:08 P.03

1178-672-403:x67

INFLAZYME

| Yanxiao ZHANG                 | Residence:         | 2120 We                               | st 44th Avenue, Suite-1 |  |  |
|-------------------------------|--------------------|---------------------------------------|-------------------------|--|--|
| NAME OF SECOND JOINT INVENTOR |                    | Vancouve                              | er, BC                  |  |  |
| NAME OF SECOND JOINT INVESTOR | V6M 2G2 CANADA CAX |                                       |                         |  |  |
|                               | Citizen of:        | People's                              | Republic of China       |  |  |
| Signature of Inventor         | Post Office /      | Address:                              | Same As Above           |  |  |
|                               |                    |                                       |                         |  |  |
| Dai <del>o</del>              |                    |                                       |                         |  |  |
|                               | Residence:         |                                       |                         |  |  |
| NAME OF THIRD JOINT INVENTOR  |                    |                                       |                         |  |  |
|                               | Citizen of:        |                                       |                         |  |  |
| Signature of Inventor         | Post Office        | Address:                              |                         |  |  |
|                               |                    |                                       |                         |  |  |
| Date                          |                    |                                       |                         |  |  |
| ·                             | Residence:         |                                       |                         |  |  |
| NAME OF FOURTH JOINT INVENTOR |                    | · · · · · · · · · · · · · · · · · · · |                         |  |  |
|                               | Citizen of:        |                                       |                         |  |  |
| Signature of Inventor         | Post Office        | Address:                              | -                       |  |  |
|                               | <u></u>            |                                       |                         |  |  |
| Date                          |                    | ·                                     |                         |  |  |

Docket No.: 1908-006-27

# Destaration, Power of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

|                          | CHROMA  | TOGRAPHIC DETERMINA<br>REACTIVE LI  | TION OF P-GLYCOPRO<br>GANDS  | TEIN     |           |               |   |
|--------------------------|---|---|--|----------|-----------|---------------|---|
| he specifica             | tion of which   |   |  |          |           |               |   |
|                          | is attached he  |   |  |          |           |               |   |
| •                        | was filed as A  | pplication Serial No. 10/009  | 344  |          |           |               |   |
|                          |   | and amended on  |  |          |           |               |   |
|                          | was filed as I  | PCT international application   |  |          |           |               |   |
|                          | Number  | PCT/US00/15820  |  |          |           |               |   |
|                          | OR  | June 9, 2000  |  |          |           |               |   |
|                          | and was amer  | ided under PCT Article 19   |  |          |           |               |   |
|                          |   |   | (if applicable).   |          |           |               |   |
| including:  W as defined | the claims, as among the claims, as among the claims, as among the claims of the claims of the claims of the country other that | that we (I) have reviewed and ended by any amendment refer the duty to disclose information of Title 37 Code of Federal Raim foreign priority benefit inventor's certificate, or \$36 in the United States, listed be ent or inventor's certificate, or hydrority is claimed. Prior F | tion known to be material to egulations.  s under 35 U.S.C. §119(a) (5(a) of any PCT International applications and have also identified applicational applicational applications. | the pate | or §365   | (b) of twhice | his application of any foreign h designated |
| least one                | oplication for pat<br>plication on which  | ant or inventor's certificate, c<br>h priority is claimed. Prior F  | oreign Application(s)  |          |           |               |   |
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| foreign an               | oplication for pat<br>plication on whice<br>application No.   | h priority is claimed. Prior F  | oreign Application(s)  |          |           |               | ned<br>No                                   |
| foreign an               | plication on which  | h priority is claimed. Prior F  | oreign Application(s)  | F        | riority ( | Clain         | ned<br>No<br>No                             |
| foreign and of the app   | plication on which  | h priority is claimed. Prior F  | oreign Application(s)  |          | riority ( | Clain         | ned<br>No                                   |

Apr 8 2002 12:08 P.02

Fax:604-279-8711

INFLAZYME

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| YAMB OF SECOND JOINT INVENTOR |             | Versoonver. |                         |
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| Vansono Thuns                 | Citizen of: | People's    | Republic of China       |
| Signature of Inventor         | Post Office | Address:    | Same As Above           |
| Anr. 2 8, 2001                |             |             |                         |
| Date                          |             |             |                         |
|                               | Residence:  |             |                         |
| NAME OF THIRD JOINT INVENTOR  |             |             |                         |
|                               | Citizen of: |             |                         |
| Signature of Inventor         | Post Office | Address:    |                         |
|                               |             |             |                         |
| Date                          |             | ٠           |                         |
|                               | Residence:  |             |                         |
| NAME OF FOURTH JOINT INVENTOR |             |             |                         |
|                               | Citizen of: |             |                         |
| Signature of Inventor         | Post Office | Address:    |                         |
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| Dete                          |             |             |                         |